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# Carboxymethyl starch: Chitosan monolithic matrices containing diamine oxidase and catalase for intestinal delivery

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ABSTRACT

The capacity of carboxymethyl starch (CMS):Chitosan monolithic tablets to protect diamine oxidase and/or catalase therapeutic enzymes against simulated gastric fluid (SGF) and to control their delivery in simulated intestinal fluid (SIF) was investigated. Enzyme formulations loaded with grass pea seedlings diamine oxidase (PSDAO) vegetal extract, catalase, or PSDAO associated to catalase, were obtained by direct compression. The CMS:Chitosan (1:1) matrix afforded a good gastric protection to PSDAO and to catalase, when each enzyme was formulated separately. Variable amounts of DAO were delivered in the SIF containing pancreatin, with maximal release reached at about 8 h, a time convenient for tablets to attain the colon. Up to 50% of the initial enzymatic activity of catalase formulated with CMS:Chitosan was found after 8 h in SIF. For the CMS:Chitosan tablets of bi-enzymatic formulations containing PSDAO:Catalase, the releases of DAO and of catalase were synchronized. The hydrogen peroxide (product of DAO activity) was decomposed by the catalase liberated in the same SIF environment. The proposed formulations could allow novel therapeutic approaches for the treatment of inflammatory bowel diseases, intestinal cancers or pseudo-allergic reactions.

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## 1. Introduction

The diamine oxidase (DAO, EC 1.4.3.6), also called histaminase (Mondovi et al., 1964), catalyzes the oxidative deamination of histamine and other biogenic amines (Mondovi et al., 1989), with the release of the corresponding aldehydes, hydrogen peroxide ($H_2O_2$) and ammonia ($NH_3$). As previously shown with other copper oxidases, such as ceruloplasmin and bovine serum amine oxidase with antioxidant, cardioprotective and antifibrillatory properties (Atanasiu et al., 1995; Mateescu et al., 1997; Mateescu and Nadeau, 2009; Mondovi et al., 1997), a vegetal DAO, with an enzymatic activity higher than that of the animal one, presents some beneficial effects in cardiac anaphylactic response (Masini et al., 2002), in myocardial ischemia and reperfusion injury (Masini et al., 2003) and in the treatment of asthma-like reaction (Masini et al., 2004). Recently, Masini et al. (2007) showed that pea seedling DAO, when parenterally administered, may have protective effects on intestinal ischemia, by reducing local tissue inflammation and by counteracting free radical-mediated tissue injury.

The DAO is the main histamine degrading enzyme acting predominantly in the intestinal tract which may protect against the endogenous (Beaven, 1982) or exogenous (food) histamine (Taylor, 1986). Food-induced histaminosis can generate plasma histamine elevation and hemodynamic alterations, particularly when associated with low DAO activity (Sattler et al., 1988). A high histamine content in some food can become toxic, especially in oriental food (Chin et al., 1989). Elevated histamine content in food and beverages can also be associated to microbial contamination (Bodmer et al., 1999). The mechanism of pseudo-allergic reactions of histamine caused by food seems to be mainly ascribed to an intestinal hyperpermeability (caused by irritant species as alcohol) or by decrease of DAO enzymatic activity. Since DAO is a major catabolic enzyme for histamine in humans, its lower level at the sites of mucosal inflammation would generate an accumulation of the released histamine, which may enhance acute

*Abbreviations:* CMS, carboxymethyl starch; GDH, L-glutamate dehydrogenase; IBD, inflammatory bowel disease; KGA, *alpha*-ketoglutaric acid; MW, molecular weight; NADPH, nicotinamide adenine dinucleotide phosphate; OCT, Organic Cation Transporter; OPDA, *ortho*-phenylenediamine dihydrochloride; PSDAO, grass pea seedlings diamine oxidase; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

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inflammatory responses (Owen, 1987). Thus, in the pathogenesis of inflammatory bowel disease (IBD), mucosal alterations are frequently related to changes of mucosal DAO activity and, respectively, in mucosal histamine content (Fox et al., 1990). In areas of macroscopically inflamed tissue in IBD, the DAO activity was found lower than in normal intestinal tissues (Schmidt et al., 1990). Furthermore, the jejunal secretion of histamine was reported as higher in Crohn's disease and was significantly correlated with the Crohn's disease activity index (Knutson et al., 1990). During intestinal post-ischemic reperfusion, it was found a marked histamine release by the intestinal mucosa, and the activity of DAO was significantly reduced (Kusche et al., 1981). High histamine content was also found in experimental neoplasias, such as colon carcinomas (Cricco et al., 1994), and a direct relationship between DAO activity and tumor progression was shown (Kusche et al., 1988). Antitumoral effects were described for pig kidney DAO (Mondovi et al., 1982) and for amine oxidases (Toninello et al., 2006).

The information concerning the therapeutical effects of DAO on IBD is rather limited. Fogel and Lewinski (2006) showed a possible therapeutic effect of hog kidney DAO, administered intraperitoneally, on a model of ulcerative colitis in rat, with a reduction of inflammatory reaction. Decreasing the histamine levels and the oxidative stress in intestinal inflammation could be a promising therapeutic strategy to better manage the IBD. As for most oxidases, a by-product of the DAO enzymatic reaction is $H_2O_2$, a pro-oxidant agent which can present desirable bactericidal and undesirable oxidative damaging effects. Catalase (EC 1.11.1.6) is an antioxidant enzyme that specifically catalyzes the decomposition of $H_2O_2$. Due to its $H_2O_2$ decomposition capacity, catalase was proposed for use in post-ischemic reperfusion injury in myocardial infarction and stroke, burns, trauma (Phillips and Snow, 1994), as parenteral formulations of the enzyme covalently bound to polyethylene glycol.

We are now proposing an oral enzymatic therapy based on PSDAO associated with catalase for the treatment of various colon diseases. The PSDAO would control the levels of histamine and would have some antioxidant effects. When associated, catalase will particularly eliminate the $H_2O_2$ by-product of DAO, preventing the local intestinal oxidative stress. It was recently found that $H_2O_2$ can inhibit copper amine oxidases and that the catalase protects amineoxidases from inactivation by produced $H_2O_2$ (Pietrangeli et al., 2004). In our case, associated catalase not only will protect PSDAO, but, in addition, could generate more oxygen ($O_2$), a substrate of PSDAO, enhancing its efficiency.

A major challenge of oral pharmaceutical forms is to deliver bioactive agents to colon without gastric or intestinal degradation during the gastro-intestinal transit of the oral dosages, and this fact constitutes an important step for our proposed therapy. As colonic microflora produce a large number of degrading enzymes, several natural polysaccharides from algal (alginates), plant (pectin, guar gum), microbial (dextran, xanthan gum) or animal origin (chondroitin), have been investigated as carriers for colon-specific drug delivery. Chitosan, a poly β-(1,4)-linked 2-amino-2-deoxy-D-glucose, insoluble at the pH of intestinal fluids, appears as an interesting excipient for site-specific delivery to the colon due to its susceptibility to glycosidic hydrolysis by microbial enzymes in the colon (Zhang and Neau, 2002). The association of cationic Chitosan with other biodegradable polymers, such as anionic CMS (Calinescu et al., 2005, 2007), could also represent an interesting way to deliver bioactive agents to the colon. Recently, monolithic tablets based on CMS:Chitosan excipients were proposed for colon delivery of small molecules (Leonida and Mateescu, 2006) and probiotics (Calinescu and Mateescu, 2008). The aim of this study was to investigate the potential of CMS:Chitosan matrix to protect the enzymes PSDAO and/or Catalase against simulated gastric conditions and to delay their delivery in simulated intestinal conditions.

## 2. Materials and methods

### 2.1. Materials

High amylose starch (Hylon VII) was obtained from National Starch (Bridgewater, NJ, USA) and Chitosan (600 kDa, 92% degree of deacetylation) from Marinard Biotech (Rivière-au-Renard, Qc, Canada). Bromocresol green (sodium salt), 1,4-diaminobutane dihydrochloride (putrescine), *ortho*-phenylenediamine dihydrochloride (OPDA), horseradish peroxidase (type I, 96 purpurogallin enzyme units/mg solid), ammonia assay kit, hydrogen peroxide (30%), Folin-Ciocalteu Reagent, pepsin (from porcine gastric mucosa: 882 units/mg protein), pancreatin (from porcine pancreas, USP specifications) and catalase (from bovine liver: 2950 units/mg solid; 4540 units/mg protein) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). The other chemicals were reagent grade and used without further purification.

### 2.2. Preparation of diamine oxidase vegetal extract from seedlings of Lathyrus sativus (grass pea)

The PSDAO was obtained from 500 g of freshly collected shoots of etiolated *L. sativus* seedlings which were homogenized in a Waring blender with 1 L of 30 mM $NaH_2PO_4$ (final pH 4.4), and then filtered. The solid residue, mainly constituted by cell walls and vascular fibers, was washed with the same buffer and the enzyme was finally eluted from the solid residue with 500 mL of 0.1 M sodium phosphate buffer (pH 7) and, then, centrifuged. The supernatant containing the DAO was lyophilized, obtaining thus the DAO vegetal extract powder, hereto called PSDAO.

### 2.3. Carboxymethyl high amylose starch excipient synthesis

The CMS excipient was synthesized in alkaline medium, as previously described (Calinescu et al., 2005, 2007), using monochloroacetic acid as starch substitution agent. Practically, an amount of 70 g high amylose starch (Hylon VII) was suspended in 170 mL of distilled water and warmed at 50 °C under continuous stirring in a Hobart planetary mixer. A volume of 235 mL of an aqueous 1.45 M NaOH solution was added and the reaction medium was homogenized for 20 min at 50 °C for gelatinization. To transform the starch into a more reactive alkoxide form (and thus favorising the starch nucleophilic substitution), 55 mL of 10 M NaOH solution were also added to the reactional medium. Then, 45.5 g of monochloroacetic acid, rapidly dissolved in a minimal volume of water, were added to the starch and the reactional medium was kept for 1 h at 50 °C for the reaction of substitution. After 1 h, the gel-slurry was neutralized with an acetic acid solution and precipitated with acetone, and the salt ions were removed by several repeated washings with acetone/water (60:40, v/v). Finally, the CMS gel-slurry was dried with acetone and kept overnight at room temperature.

The polymer powder was then sieved and particles with granulometry less than 300 μm were retained for the formulation study. Fourier transform infrared spectroscopy was used to detect the presence of newly introduced carboxymethyl groups. The degree of substitution was determined by direct titration as previously described (Calinescu et al., 2005).

### 2.4. Preparation of Chitosan excipient

The powder of Chitosan was prepared as previously described (Calinescu and Mateescu, 2008), by dissolving the purchased Chitosan in an acetic acid solution (2%), followed by its filtration (to eliminate impurities). Then, a solution of NaOH (1 M) was added

to the homogeneous filtrate until a pH of 6.5. The gel-slurry was precipitated with 100% acetone, thoroughly washed with distilled water, and finally dried at room temperature. Excipient fraction smaller than 300 μm was retained for the preparation of monolithic tablets.

### 2.5. Fabrication of monolithic tablets

The powders of CMS and/or Chitosan excipients were well mixed with the bioactive agents (PSDAO and/or catalase powders) until homogenization. Flat-faced tablets of 300 mg were obtained by direct compression (2.5 T) of the powder mixtures using 9 mm cylinder outfits and a Carver press (Wabash, IN, USA). The PSDAO powder (30% loading) was formulated with CMS alone, CMS:Chitosan (1:1, w/w) or Chitosan alone. Catalase (3.3%, 10%, 20%, 50% loading) was formulated only with CMS:Chitosan (1:1, w/w). Tablets based on 100% enzymes (excipient-free) were also produced as control. Bi-enzymatic monolithic tablets, based on CMS:Chitosan (1:1, w/w) and containing both enzymes (PSDAO and catalase), were also realized with a constant loading of PSDAO (20%) and a variable loading of catalase (10% and 20%) per tablet.

### 2.6. Gastric stability of tablet formulations (with or without PSDAO or catalase)

Tablets of 300 mg, based on CMS:Chitosan (1:1) with 0% and 50% enzyme loading or on 100% enzyme (without excipients), and loaded with 5% bromocresol green (15 mg pH indicator per tablet), were prepared as previously described. Each tablet was incubated 60 min in 50 mL of SGF containing 0.32% pepsin (USP, 2009) at 37 °C and 50 rpm (incubator shaker, series 25D, New Brunswick Scientific Co., NJ, USA). The tablet integrity and color modifications were noticed on the whole and on cross-sectioned tablets.

### 2.7. Stability of enzyme formulations in simulated gastric fluid

Tablets based on CMS alone, CMS:Chitosan (1:1) or Chitosan alone (as excipients), containing 30% PSDAO (as active principle ingredient), were incubated for 0, 30, 60, 120 min in 50 mL SGF, pH 1.2, with 0.32% pepsin (USP, 2009), at 37 °C and 50 rpm using the incubator shaker as before. Also, the CMS:Chitosan formulations containing catalase only (different loadings) or the two enzymes (PSDAO:Catalase) were incubated for 60 min in SGF (in the same conditions as before). The remaining enzymatic activities in the tablets were determined after the incubation times indicated above by crushing the tablets in 50 mL of 50 mM potassium phosphate buffer (pH 7.0), followed by sample filtration.

### 2.8. Enzyme delivery in simulated intestinal fluid

The tablet formulations containing PSDAO only, catalase only or the two enzymes were first incubated in SGF for the same incubation times indicated above. After SGF incubation, tablets were individually transferred into 50 mL SIF, pH 6.8, with 1% pancreatin (USP, 2009) and incubated at 37 °C and 50 rpm (total dissolution time of 24 h), using the same incubator shaker. Samples of 1 mL were taken from SIF after regular intervals of time, filtered and the DAO and catalase enzymatic activities were determined (as described at Section 2.10).

### 2.9. Evaluation of DAO enzymatic activity in the presence of catalase

PSDAO:Catalase powders at different weight ratios (6:1, 2:1, 1:1) were dissolved in 50 mL phosphate buffer solution (50 mM, pH 7.0), kept 120 min under agitation at 4 °C and filtered prior to determine the DAO enzymatic activity in the absence or in the presence of catalase.

### 2.10. Determination of DAO enzymatic activity

(a) Enzymatic assay of DAO with the peroxidase coupled reaction (specific for released $H_2O_2$). The DAO enzymatic activity was spectrophotometrically assayed with a peroxidase coupled reaction, in the same conditions as previously described (Calinescu et al., 2010). Briefly, the reactional mixture containing 640 μL of 50 mM potassium phosphate buffer (pH 7.0), 10 μL of peroxidase solution (0.1 mg/mL), 50 μL of 30 mM OPDA solution and 200 μL of 30 mM putrescine solution was incubated for 5 min at 37 °C and then, 100 μL of DAO samples were added to start the dosage. The enzymatic reactions were conducted at 37 °C for 10 min, when 100 μL of HCl (4 M) were added and the final absorbance was read at 484 nm using a Beckman DU®-6 spectrophotometer. The standard curve was prepared with serial concentrations of $H_2O_2$ from 0 to 68 μM.

(b) Enzymatic assay of DAO with the L-glutamate dehydrogenase (GDH) coupled reaction (specific for released $NH_3$). In particular case of the association of DAO with catalase, the DAO assay *via* peroxidase cannot apply due to decomposition of $H_2O_2$ by the associated catalase. For these cases, the DAO enzymatic activity was also evaluated using an ammonia assay kit (Sigma–Aldrich), where $NH_3$ released from the putrescine substrate (under DAO catalysis) reacts with *alpha*-ketoglutaric acid (KGA) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GDH. The reactional mixture containing 1 mL of assay kit reagent (KGA and NADPH), 200 μL of 30 mM putrescine solution, 10 μL of GDH (kit reagent) was incubated for 5 min at 37 °C and, finally, 100 μL of DAO samples (containing or not catalase) were added. The enzymatic reactions were conducted at 37 °C for 10 min, monitoring the decrease of absorbency at 340 nm. Since catalase also strongly binds NADPH in its active center, the catalase interference was subtracted from each determination of DAO enzymatic activity.

One enzymatic unit (EU) of DAO was defined as the amount of enzyme catalyzing the oxidation of 1.0 μmole of putrescine per 10 min at pH 7.0 and 37 °C.

For CMS, CMS:Chitosan (1:1) and Chitosan formulations containing 30% PSDAO only, the DAO enzymatic activity was determined by the peroxidase coupled assay.

For the formulations based on CMS:Chitosan (1:1) and containing 20% PSDAO and different loadings in catalase (0%, 10% and 20%), the DAO enzymatic activity was determined by the peroxidase coupled assay and by the GDH coupled assay.

The DAO enzymatic activity of vegetal extract powder (PSDAO) and of different weight ratios of PSDAO:Catalase powders, was also evaluated by the two enzymatic reactions, as proposed before.

### 2.11. Determination of catalase enzymatic activity

The enzymatic activity of catalase was determined spectrophotometrically (Beckman DU®-6) by monitoring at 240 nm the $H_2O_2$ decrease during catalysis (Claiborne, 1985). The reactional medium contained: 2.9 mL of $H_2O_2$ (0.036%) prepared in 50 mM potassium phosphate buffer (pH 7.0) and 0.1 mL of filtered sample containing catalase.

One enzymatic unit (EU) of catalase is defined as the amount of enzyme decomposing 1.0 μmole of $H_2O_2$ per minute at pH 7.0 and 21 °C, while the $H_2O_2$ concentration falls from 10.3 mM to 9.2 mM.

Protein concentrations of the PSDAO and of catalase powders were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

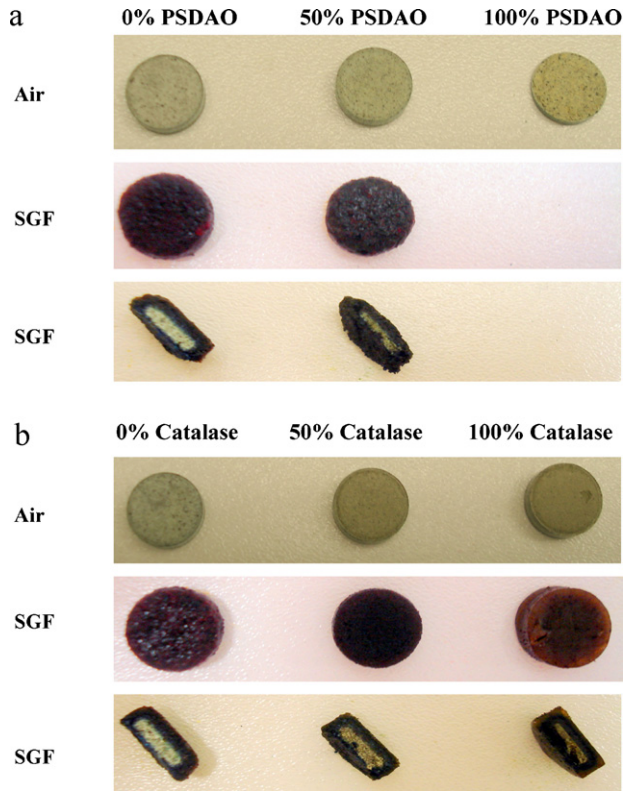



**Fig. 1.** pH stability of enzyme formulations in tablets based on CMS:Chitosan. Monolithic tablets based on (a) CMS:Chitosan (1:1) with 0% and 50% enzyme loading and on excipient-free 100% diamine oxidase vegetal extract (PSDAO) or (b) catalase, containing bromocresol green (pH indicator). Untreated (air) or incubated tablets for 60 min in simulated gastric fluid containing pepsin, at 50 rpm and 37 °C (whole and cross-sections of tablets). The blue color indicates protection against gastric acidity. Substitution degree of CMS: 0.126.

## 3. Results and discussion

### 3.1. Gastric stability of tablet formulations (with or without PSDAO or catalase)

The hydrophilic matrix based on binary mixtures of CMS:Chitosan (1:1), in which both macromolecular excipients contribute to a physical and chemical stabilization of the dosage form (Calinescu and Mateescu, 2008; Leonida and Mateescu, 2006), seems a good system for the formulation of orally administrable therapeutic proteins, such as PSDAO and catalase. To evaluate the pH within the tablets, the test with a pH indicator is useful because it indicates in which extent the excipients can protect the active agent(s) in SGF following the color change of the pH indicator (*i.e.*, for bromocresol green pH indicator, the color changes from blue, over pH 5.4, to yellow-orange, below pH 3.8). Thus, to the air, before incubation in SGF, the dry tablets based on CMS:Chitosan (1:1) unloaded (0%) and loaded with 50% PSDAO or with catalase, and the tablets based on 100% enzyme (PSDAO or catalase) presented some blue points at the external surface, corresponding to the pH indicator (Fig. 1a, b). The surface of the entire tablets based on CMS:Chitosan excipients (with 0% and 50% enzyme loading) was blue during the 60 min of acidic incubation (SGF). The CMS:Chitosan matrix forms an outer gel barrier, affording thus a certain protection against gastric acidity, despite the fact that the tablets are not coated with gastro-protective materials. At the surface of the whole tablets, some small orange regions were also detected, corresponding to a certain presence of acidity (mostly limited at the very outer layer).

The CMS:Chitosan formulations with PSDAO only were less stable at 50% loading and unstable when formulated without CMS:Chitosan (100% PSDAO). Thus, the excipient-free formulation, based on 100% PSDAO only, was rapidly disintegrated and dissolved during the incubation in SGF medium. This lower stability of PSDAO tablet could be related to a certain amount of phosphate in the powder extract remaining after the preparation procedures. On the other hand, the presence of hydrophilic phosphate in PSDAO preparation can be an advantage for formulation, ensuring the tablet hydration and the release of loaded enzyme.

Differently, the excipient-free tablets, based on 100% catalase, were compact after the SGF incubation, with no tendency of swelling nor disintegration, but exhibiting an orange external surface of the tablets, showing lesser protection in acidic medium. When the tablets were cross-sectioned, the core of the tablets was dry, with no gastric fluid inside the tablets and, consequently, no solubilization of the bromocresol green particles. Furthermore, a peripheral blue layer (pH greater than 5.4) close to the surface of the tablets was found (Fig. 1a, b). Thus, the presence of the proteins in the formulations may also contribute to gastro-protection, as found for catalase at loading of 50% and 100%. These aspects are useful and important for further enzyme formulations, showing that not only the CMS:Chitosan excipients afforded a certain gastro-protection of bioactive agents, but protein itself can generate an outer protective gel layer keeping dry the core of the tablets.

### 3.2. Stability of enzyme formulations in simulated gastric fluid and enzyme delivery in simulated intestinal fluid

The PSDAO was found as very sensitive to gastric acidity and to pepsinolysis, loosing its entire enzymatic activity in less than 15 min of incubation, either in the presence or absence of pepsin. Differently, the DAO maintains a certain stability at proteolysis to pancreatin (Calinescu et al., 2010), and this is an important aspect for further therapeutic applications of the enzyme when released in intestinal fluids.

Formulations based on CMS, CMS:Chitosan (1:1) or Chitosan and containing 30% PSDAO presented a certain stability in SGF conditions (pH 1.2, pepsin) during the whole periods of incubation. After 60 min of gastric treatment, CMS:Chitosan (1:1) and Chitosan formulations afforded a better protection of DAO than CMS (Fig. 2a) and after 120 min of incubation, the CMS:Chitosan matrix presented a better efficacy in term of protection of PSDAO, with a 55.5% remaining DAO activity found inside the tablet (Fig. 2a). Thus, the CMS:Chitosan matrix could present a potential interest, ensuring a good protection of the DAO even after 120 min incubation in SGF, with maximal release of therapeutic enzyme in SIF at about 8 h (enough for tablets to reach the colon). The release of bioactive agent can be controlled by an adequate choice of the amount and of the molecular weight of Chitosan in the tablet (Calinescu and Mateescu, 2008). Thus, for the Chitosan of 600 kDa (the present study), a delay in the DAO liberation was already observed after 30 min of gastric incubation of CMS:Chitosan (1:1) tablets compared to those based on CMS matrix only, which released rapidly the bioactive agent in SIF. In the case of CMS, the release time can be modulated by alpha-amylase from pancreatin, which can still act on CMS excipient (Calinescu et al., 2005). This is an important advantage of CMS as excipient. The tablets based on CMS only are not suitable for colon delivery because of their fast dissolution in SIF medium containing pancreatin.

For better understanding of mechanisms controlling the DAO release, the tablets were also incubated in SIF only, without previous incubation in SGF. When incubated directly in SIF, the absence of the external gel of Chitosan generated a faster liberation of DAO for CMS:Chitosan and Chitosan formulations (Fig. 2b) in comparison with the same formulations previously incubated in SGF (30, 60,

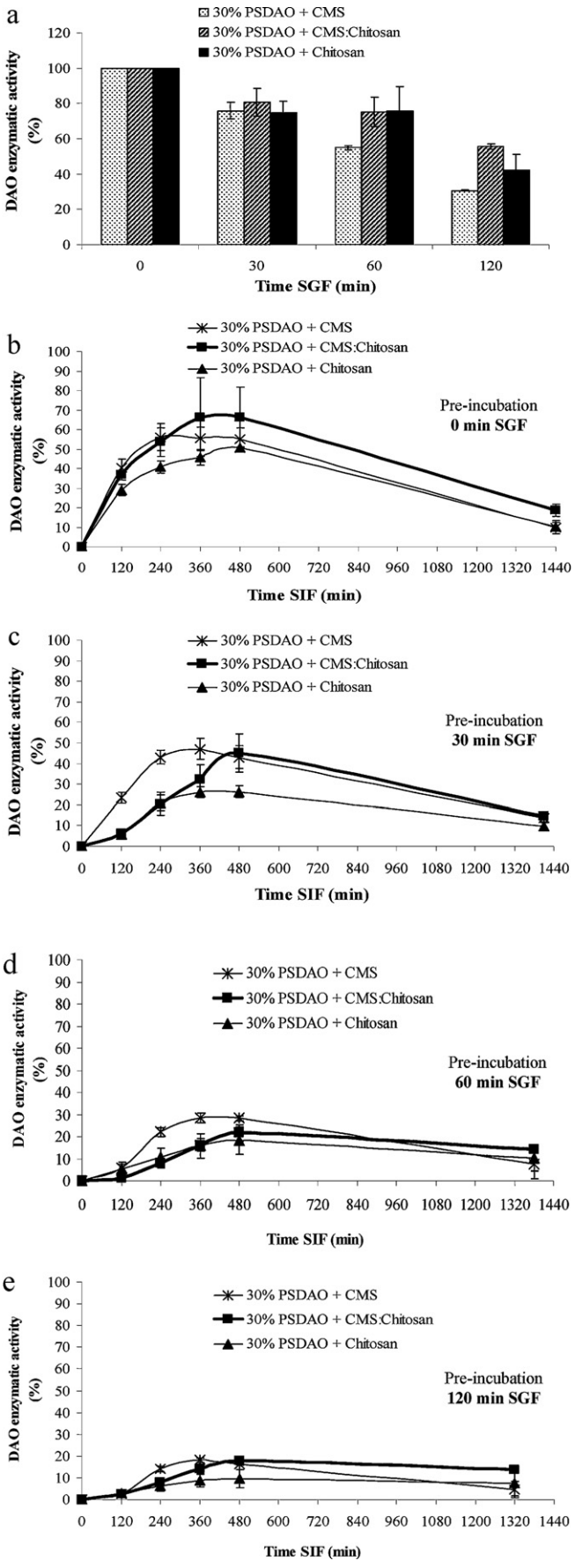



**Fig. 2.** Gastric stability and intestinal delivery of vegetal diamine oxidase (PSDAO) in different formulations. Monolithic tablets based on CMS (substitution degree: 0.126), CMS:Chitosan (1:1) or Chitosan and containing 30% PSDAO were incubated up to 120 min in simulated gastric fluid (SGF with pepsin). (a) DAO remaining enzymatic activity (%) inside the tablets, as determined after different periods of SGF treatment. The liberation of DAO (%) from monolithic tablets in simulated intestinal fluid (SIF with pancreatin) after (b) 0 min, (c) 30 min, (d) 60 min and (e) 120 min of SGF incubation (50 rpm and 37 °C). The DAO activity is expressed in percentages, considering as 100% the values determined in 50 mL phosphate buffer at 0 min. For DAO formulated with CMS, the 100% was 0.45 ± 0.032 EU/mL sample, 0.41 ± 0.019 EU/mL sample for CMS:Chitosan or 0.40 ± 0.03 EU/mL sample for Chitosan. $n = 3$.

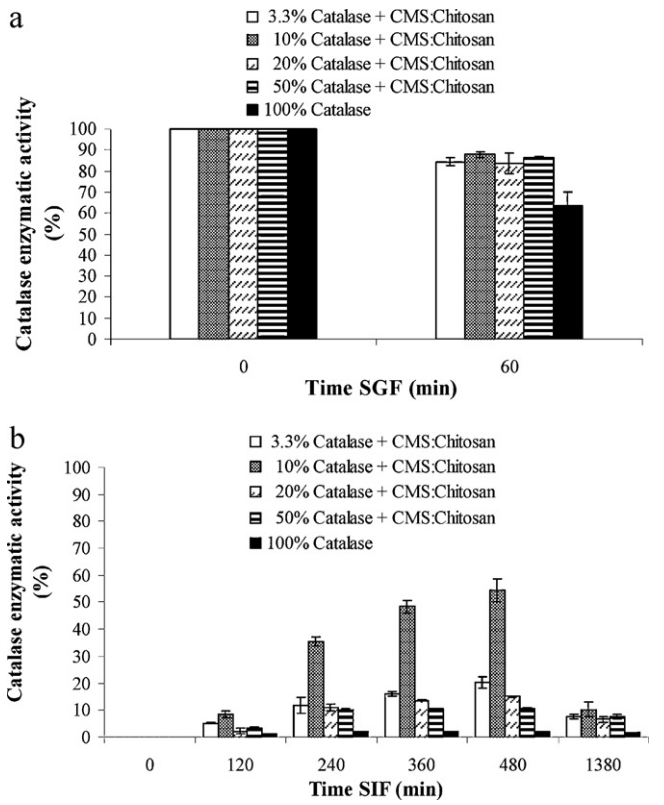



**Fig. 3.** Gastric stability and intestinal release of catalase at different loadings formulated with CMS:Chitosan. Monolithic tablets of CMS:Chitosan (1:1) with increasing loading of catalase and of excipient-free (100%) catalase were first incubated for 60 min in simulated gastric fluid (SGF with pepsin) at 50 rpm and 37 °C. Substitution degree of CMS: 0.126. The enzymatic activity of catalase (%) was evaluated (a) after 60 min of gastric incubation of tablets and (b) during the liberation in simulated intestinal fluid (SIF with pancreatin), after previous incubation of 60 min in SGF. Catalase activity was expressed in percentage, considering for each loading, as 100%, the catalase activity formulated with CMS:Chitosan determined in 50 mL phosphate buffer at 0 min. $n = 3$.

120 min), where the outer gel delayed the DAO delivery (Fig. 2c–e). The formulation based on CMS:Chitosan (1:1) presented a better delivery of DAO than the Chitosan formulation (Fig. 2b–e). In an acidic environment, Chitosan tablets form gels (Wang et al., 2011) due to their amino groups, contributing to the protection of active agent and, at the same time, allowing its slow-release. Wang et al. (2011) realized a study of Nuclear Magnetic Resonance imaging on monolithic tablets of Chitosan showing the formation of a transparent gel thick layer around the hard core. The size of the tablet core decreased gradually with time, and no proton signal was detected in the dry core during the process, as shown by the clear-cut feature of the proton density profile (Wang et al., 2011). This gel layer formed in an acidic medium contributes to delay the liberation of DAO from Chitosan and CMS:Chitosan monolithic tablets.

The second therapeutic enzyme used in this study, catalase, was first formulated alone with CMS:Chitosan (1:1) at different loadings. It was observed that, at increasing catalase loading from

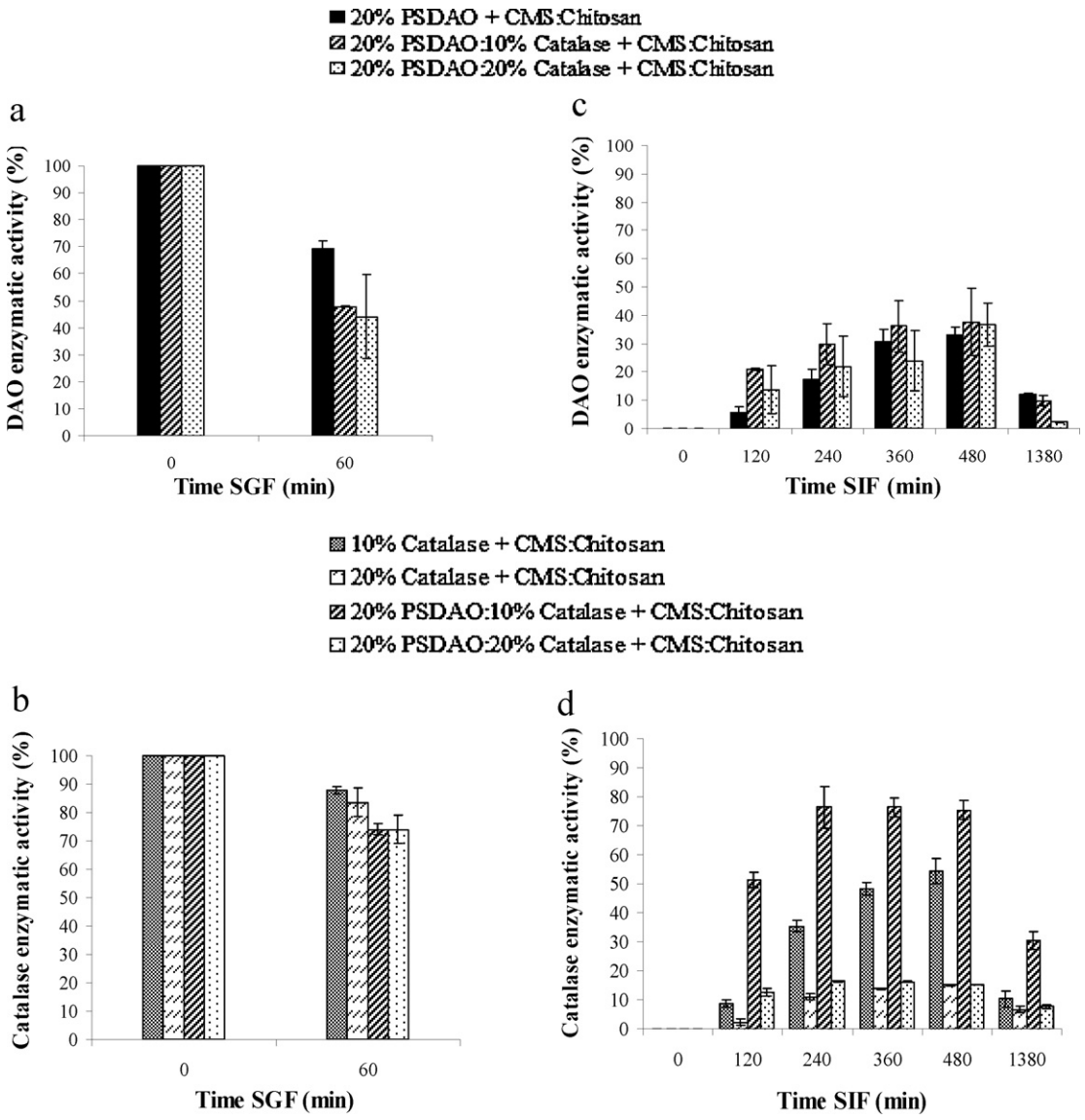



**Fig. 4.** Mono- and bi-enzymatic CMS:Chitosan formulations containing vegetal diamine oxidase (PSDAO) and/or catalase. Gastric stability in simulated gastric fluid (SGF) containing pepsin (0 and 60 min) of (a) PSDAO and (b) catalase as mono- and bi-enzymatic formulations based on CMS:Chitosan (1:1) and (c, d) their liberation in simulated intestinal fluid (SIF) containing pancreatin, after 60 min tablet incubation in SGF. Substitution degree of CMS: 0.126. The enzymatic activity of DAO (20% loading: 0.39 ± 0.001 EU/mL sample) and of catalase (10% and 20% loading), formulated as mono- and bi-enzymatic CMS:Chitosan tablets, and determined in 50 mL phosphate buffer (0 min), was considered as 100%. $n = 3$.

3.3% to 50%, its residual enzymatic activity found inside the tablet remained relatively constant (more than 80%) after 60 min of SGF incubation, suggesting that the catalase can undergo a kind of self-protection by possible protein–protein interactions, in addition to the relative protection afforded by the excipients (Fig. 3a). Thus, due to possible intra- and inter-chain protein associations, catalase generated an outer protective gel layer, preventing the gastric fluid access into the tablet. As found by the pH indicator test, the assay of the enzyme activity showed that the formulation of 100% catalase tablets (excipient-free) presented a relatively good stability in gastric fluid (60 min), with 63% catalase remaining enzymatic activity per tablet (Fig. 3a). Differently, the free catalase solution (unformulated) was almost totally degraded in the same conditions (data not shown).

Higher loading in catalase per tablet did not deliver higher percentages of catalase in SIF medium (Fig. 3b). Only the CMS:Chitosan formulation containing 10% catalase liberated more than 50% catalase (enzymatic activity) after 480 min of SIF incubation. Differently, for the catalase loadings between 20% and 100%, less than 15% catalase enzymatic activity was released during the first 480 min in SIF (Fig. 3b). A certain ratio between catalase and polymeric excipients (CMS and Chitosan) should be kept to better protect and to delay the catalase delivery over 8 h in SIF.

Physical properties of tablets might also affect the behavior of formulations in simulated gastro-intestinal conditions. Tests of crushing strength (hardness) and disintegration were performed on similar protein formulations (monolithic tablets) based on similar CMS, CMS:Chitosan (1:1, physical mixture) and Chitosan excipients (Assaad et al., 2011). Generally, the tablets containing protein and the mentioned excipient(s) showed crushing strengths higher than the excipient-free protein tablets, indicating that the presence of excipient(s) favors the physical stabilization of tablets following compression. There more, the presence of CMS enhanced the crushing strength of tablets from 128 N with Chitosan only to 176–179 N with CMS:Chitosan mixture (Assaad et al., 2011). Regarding the disintegration properties in various media, due to the solubility of

Chitosan in an acidic medium, the disintegration time was shorter for the formulations containing higher amount of Chitosan (Assaad et al., 2011). The longest disintegration time was shown by CMS due to the stabilization of its chains by carboxyl groups in an acidic medium. In a simulated intestinal medium, the tablets based on CMS, CMS:Chitosan mixture or Chitosan showed a disintegration time of about 60–70 min, the disintegration time in an intestinal fluid being shorter for each mentioned formulation than that in a gastric medium (Assaad et al., 2011). Due to the fact that Chitosan cannot form a hydrogel in a neutral medium, the tablets based on Chitosan present a low cohesion between the particles, resulting in a faster disintegration in an intestinal medium.

In order to reduce the amount of the hazardous $H_2O_2$ by-product of DAO, the catalase, as second therapeutic enzyme, was added to the PSDAO formulation based on CMS:Chitosan. The presence of catalase in the PSDAO formulation diminished the determined DAO remaining enzymatic activity inside the tablet after 60 min of SGF incubation, either for 10% or 20% catalase when detected with the glutamate dehydrogenase coupled assay (Fig. 4a). A reduction of remaining catalase activity, after 60 min in SGF, was also observed for the formulations containing the PSDAO (Fig. 4b). For the bi-enzymatic formulations with CMS:Chitosan, the presence of 10% or 20% catalase did not significantly modify the DAO liberation (Fig. 4c). Differently, for the same formulations, the presence of 20% PSDAO preparation increased the catalase synchronously liberated in SIF medium (Fig. 4d). Thus, the presence of PSDAO in the bi-enzymatic formulations contributed to a better liberation of catalase, probably, due to the presence of phosphates in the PSDAO vegetal extract which enhance the dissolution. The CMS:Chitosan formulation containing 20% PSDAO:10% Catalase liberated more catalase than the bi-enzymatic formulation containing 20% PSDAO:20% Catalase, where the protein–protein interactions were probably more representative.

Using the peroxidase coupled assay, an apparent decrease of DAO enzymatic activity was obtained in the presence of catalase in function of PSDAO:Catalase ratios (Fig. 5a), due to the diminution of released $H_2O_2$ (substrate for catalase). Differently, using the GDH coupled assay, the DAO enzymatic activity was found constant (Fig. 5b) for the same PSDAO:Catalase ratios, because the assay measures specifically $NH_3$ and not $H_2O_2$, showing that the DAO enzymatic activity is not affected by the presence of catalase.

Vegetal DAO enzyme presents a high specificity for primary diamines and histamine, catalyzing their oxidation to corresponding aldehyde, $H_2O_2$ and $NH_3$. In the presence of putrescine as substrate for DAO, the OPDA, as co-substrate of peroxidase (Calinescu et al., 2010), is oxidized into a colored product (azo-aniline) by the released $H_2O_2$, under peroxidase catalysis (Scheme 1a). When catalase is synchronously released, it will decompose $H_2O_2$ released from DAO enzymatic activity, resulting in a decrease of the azo-aniline product of the peroxidase reaction. This alters the DAO dosage and apparently decreases the measured DAO activity (Scheme 1a). When determined by the GDH coupled assay (specific for $NH_3$ and not for $H_2O_2$), the DAO enzymatic activity remained constant for all the tested ratios of PSDAO:Catalase (Scheme 1b). The DAO enzymatic activity consists in oxidation of putrescine in the presence of dissolved $O_2$ (the two substrates of DAO). When catalase is also added in solution (different ratios of PSDAO:Catalase), part of $H_2O_2$ produced by DAO will be decomposed by catalase generating, thus, some supplementary $O_2$, which is expected to enhance the rate of substrate (histamine, putrescine) oxidation and even to shift the equilibrium in favor of the reaction products. From the apparent decrease of DAO enzymatic activity in the presence of catalase, it is possible to determine the number of μmoles of $H_2O_2$ decomposed by catalase. Consequently, from differences between DAO enzymatic activity of PSDAO alone and at different ratios PSDAO:Catalase, it

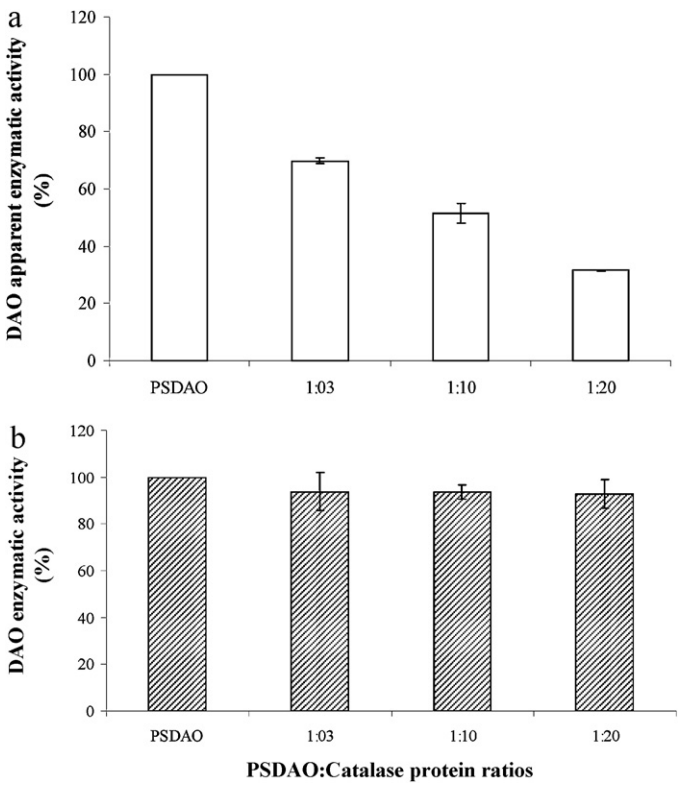
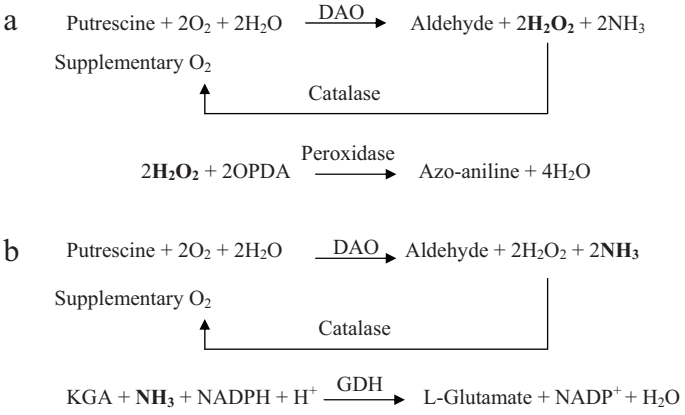



**Fig. 5.** Evaluation of diamine oxidase enzymatic activity in the presence of catalase. Diamine oxidase (DAO) enzymatic activity was evaluated (a) by the peroxidase coupled assay of the released $H_2O_2$ and (b) by glutamate dehydrogenase (GDH) coupled assay of the released $NH_3$. The DAO enzymatic activity (%) was evaluated at different mass ratios (6:1, 2:1, 1:1) of vegetal DAO (PSDAO) and Catalase powders (containing 1:3, 1:10, 1:20 PSDAO:Catalase protein ratios), which were dissolved in 50 mL phosphate buffer solution (pH 7.0). The enzymatic activities of PSDAO, determined in phosphate buffer by peroxidase coupled assay (12.81 ± 0.27 EU/mg protein) and by GDH coupled assay (13.15 EU/mg protein), in absence of catalase, were considered as 100%. $n = 3$.




**Scheme 1.** Representation of the enzymatic coupled reactions of diamine oxidase (DAO). (a) Peroxidase coupled assay, specific for released $H_2O_2$ and (b) glutamate dehydrogenase (GDH) coupled assay, specific for released $NH_3$.

is possible to estimate the additional $O_2$ produced by catalase in our *in vitro* study (0.0115, 0.0185 and 0.026 μmoles $O_2$/10 min/mL reactional medium, at 37 °C). The concentration of $O_2$ currently dissolved in water at 37 °C is of about 0.212 μmoles $O_2$/mL, which is in excess compared with the $O_2$ generated from the catalase activity. Thus, the amount of additional $O_2$ produced by $H_2O_2$ decomposition seems not high enough to enhance the DAO enzymatic activity, which remained constant in the presence of catalase. However, the

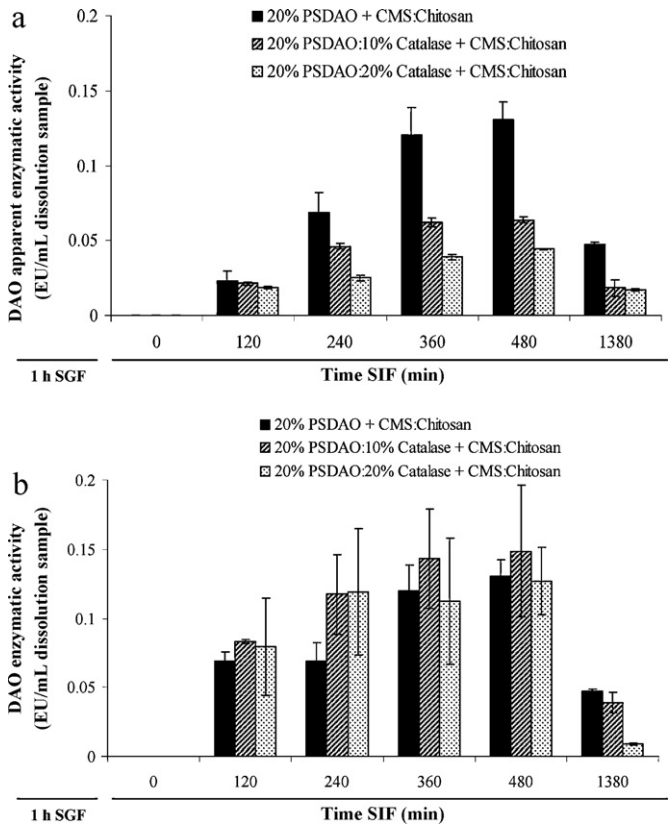



**Fig. 6.** *In vitro* evaluation of the bi-enzymatic formulation based on CMS:Chitosan. Enzymatic activity of diamine oxidase (DAO) released in simulated intestinal fluid (SIF with pancreatin), determined (a) by the peroxidase coupled assay, specific for the released $H_2O_2$, and (b) by the glutamate dehydrogenase coupled assay, specific for the released $NH_3$. Monolithic CMS:Chitosan (1:1) tablets were first incubated for 60 min in simulated gastric fluid (SGF with pepsin), followed by incubation in SIF, at 50 rpm and 37 °C. Substitution degree of CMS: 0.126. $n = 3$.

association of catalase to DAO and their simultaneously release is supposed to be therapeutically beneficial in IBD treatment, since the volume of available fluid in the colon is reduced and the $O_2$ availability is scarce. Thus, additional low amounts of $O_2$ will probably be efficient for DAO activity.

The DAO liberation in SIF from the PSDAO:Catalase bi-enzymatic CMS:Chitosan tablets, evaluated with the peroxidase coupled assay, showed an apparent reduction of released DAO measured activity, more accentuated for the formulation containing a higher loading of catalase (20% PSDAO:20% Catalase) compared to the mono-enzymatic formulation containing 20% PSDAO only (Fig. 6a). When DAO liberation in SIF was determined by the GDH coupled assay (specific for the $NH_3$ released), no significant differences were obtained between the mono-enzymatic PSDAO formulation and the bi-enzymatic formulations containing 10% or 20% catalase (Fig. 6b). The capacity of our bi-enzymatic CMS:Chitosan formulations to produce and to decompose $H_2O_2$, investigated with the two DAO enzymatic assays (Scheme 1), confirmed that the apparent decrease of DAO enzymatic activity, determined *via* peroxidase assay, was due to the decomposition of $H_2O_2$ by catalase liberated from the tablets almost in the same time with DAO. Using the GDH coupled assay, there were no differences between the activities of the released DAO formulated alone (20% loading) or as bi-enzymatic formulations, confirming the results obtained before with different ratios of the two non-formulated free enzymes. This fact is important, because, when the bi-enzymatic formulation is administrated *in vivo*, the released DAO will locally degrade the histamine (endogenous, from intestinal inflammatory reactions, or exogenous, from food) and will produce $H_2O_2$, a pro-oxidant with damaging effects. Therefore, the catalase will be beneficial, particularly when locally liberated simultaneously with DAO, to decompose the $H_2O_2$ from histamine catabolism or from other inflammatory process. The DAO appears as a bi-functional enzyme: (i) histaminase, with an amine oxidase activity controlling the level of biogenic amines, decomposing the pro-inflammatory histamine, and (ii) antioxidant, scavenging the pro-oxidant oxidative species. The presence of catalase in our mono- and bi-enzymatic CMS:Chitosan formulations has also several roles: (i) to protect DAO against $H_2O_2$ damages and to improve the stability of the formulations in simulated gastro-intestinal conditions, (ii) to afford a certain gastro-protection, based on a kind of self-stabilization by protein–protein interactions within the tablets, (iii) to reduce the amount of the hazardous $H_2O_2$, a pro-oxidant produced from DAO activity or from other inflammatory reactions.

To prevent systemic effects of histamine, rapid and efficient clearance from blood is vital. When the capacity for local histamine elimination is unbalanced, it can return, *via* Organic Cation Transporters (OCT), into the systemic circulation and generate deleterious elevations in plasma histamine level (Aschenbach et al., 2009). Oral administration of histaminase formulated with CMS and Chitosan will decrease the level of histamine in the intestinal lumen, will prevent readsorption *via* OCT and will enhance histamine bioelimination. In this context, the bi-enzymatic PSDAO:Catalase formulation with CMS:Chitosan excipients could be an alternative for a better control of the inflammation and related deleterious effects occurred in the inflammatory bowel diseases.

## 4. Conclusion

This study showed the potential of the CMS:Chitosan formulations to protect PSDAO and/or Catalase against simulated gastric conditions and to control their release in simulated intestinal fluid. Tablets loaded with PSDAO:Catalase are expected to improve the treatment of inflammatory enteric diseases by reducing local inflammation through accelerated histamine catabolism and by preventing free radical-mediated tissue injury. A possible therapeutic effect of DAO could also be expected in pseudo-allergic diseases, due to the elimination of histamine excess in intestinal lumen, and in intestinal tumors, due to the anti-neoplastic properties of amine oxidases. Our proposed formulations may open the way to innovative and non-toxic bi-functional enzyme therapeutic approaches for intestinal diseases.

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